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Cultural Characters of the Chestnut-Blight Fungus and Its Near Relatives — C. L. SHEAR and NEIL E. STEVENS

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CULTURAL CHARACTERS OF THE CHESTNUT-BLIGHT FUNGUS AND ITS NEAR RELATIVES.[1]

By C. L. Shear, *Pathologist in Charge of Small-Fruit Diseases*, and Neil E. Stevens, *Forest Pathologist.*

INTRODUCTION.

The chestnut-blight fungus *Endothia parasitica* (Murr.) And. and And. has been found to have two or three rather closely related species in this country, as follows: *Endothia radicalis* (Schw.) DeNot., *E. radicalis mississippiensis*, and *E. gyrosa* (Schw.) Fr. One or two other species have been found, but are not likely to be confused with *E. parasitica.*

Endothia radicalis (Schw.) DeNot. has already been well described by the Andersons [2] as *E. virginiana*, and also by Clinton [3] as *E. gyrosa.*

A study of the type specimen of *Sphaeria radicalis* Schw., made by the senior writer,[4] has shown that this name properly belongs to the plant having narrowly oval ascospores, usually ranging from 6 to 9 by 2.5 to 3.5 microns. In general macroscopic appearance this species very closely resembles *Endothia parasitica*, and we were at first inclined to the belief that the two were not specifically distinct, but after a careful study of hundreds of specimens, cultures, and inoculations of both species from many localities no series of intergrading forms, either morphological or physiological, has been found. At first glance the form described below might be so considered, but it is quite distinct and constant in its cultural characters. Unless such intergrading forms should be discovered later there seems to be no way of escaping the conclusion that the two organisms are specifically distinct, according to the most conservative taxonomic standard of species prevailing at present in mycology. The lack of fluctuating variations in these organisms, both in nature and in artificial cultures, is in striking contrast with the species of Glomerella, which, according to the senior writer's experience, showed few or no stable characters of any kind.

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[1] Issued July 5, 1913.

[2] Anderson, P. J., and Anderson, H. W. The chestnut-blight fungus and a related saprophyte. Phytopathology, v. 2, no. 5, pp. 204–210, 1912.

[3] Clinton, G. P. The relationships of the chestnut-blight fungus. Science, n. s., v. 36, no. 939, pp. 907–914, 1912.

[4] C. L. Shear. The type of Sphaeria radicalis Schw. To appear in Phytopathology, v. 3, no. 3, 1913.

Endothia radicalis mississippiensis subsp. nov.—The writers are giving this name tentatively to a plant of which only four collections have thus far been seen. These were all found on dead chestnut coppice in northern Mississippi. In morphological characters it does not appear to differ appreciably from *E. radicalis*, but in cultures it shows constant and easily recognized differences. In pycnospore streak cultures on potato agar+6 or +7 Fuller's scale this variety produces less aerial growth along the streak than *E. parasitica*. When 5 or 6 days old the surface growth is xanthine orange by reflected light. When 2 weeks old it shows the most marked characters, being grenadine red by transmitted light and producing no spore masses. On corn meal the color of the mycelium becomes orange chrome at the end of a week and the pycnidia are larger and less numerous than in *E. parasitica*. The perilla-purple color developed in this medium by *E. radicalis* appears very tardily or not at all in cultures of this variety. There is no evidence yet that it is any more parasitic in tendency than *E. radicalis*. More material and data are necessary to determine satisfactorily its taxonomic status.

Endothia gyrosa (Schw.) Fr.—Under this name we shall treat of the fungus which Clinton [1] calls *E. radicalis* (Schw.?) Farl. This species is easily recognized in its ascospore stage by its long, narrow, usually nonseptate ascospores, mostly measuring from 7 to 11 by 1.5 to 2.5 microns. A discussion of the identity of this species will be given in a later publication.

Since these organisms when found on dead plants are not easily distinguished from *Endothia parasitica* by macroscopic characters in the field, nor always with certainty from microscopic studies, especially when only the pycnidial fructifications are found, it is desirable to have some other certain means of diagnosis in case of doubt. With this end in view the cultural characters and reactions of the organisms have been studied on various culture media. Over 2,000 cultures have been studied, including 19 different collections of *E. parasitica* selected from 100 or more, mostly from Castanea species, and a few from Quercus, ranging from New Hampshire to southern Virginia, 24 European collections of *E. radicalis* on *Castanea sativa* from Italy and Switzerland, and 54 American specimens taken from over 100 collections on Quercus species and *Castanea dentata*, ranging from southeastern Pennsylvania and Ohio to South Carolina and Alabama; also 4 collections of the Mississippi form on *Castanea dentata* from northern Mississippi and 34 collections of *E. gyrosa* selected from 140 on Quercus species, Liquidambar, Fagus, and *Castanea dentata* from Connecticut and Michigan to Florida and Texas.

[1] Clinton, G. P. Chestnut-bark disease. Connecticut Agricultural Experiment Station Annual Report, 1912, pt. 5, p. 422, 1913.

PREVIOUS CULTURE WORK.

Clinton, the Andersons, Pantanelli, and Fulton have already made contributions to this subject. Clinton,[1] 1908, describes cultures of *Endothia parasitica* on Lima-bean agar. Pycnospores were produced and the growth gradually changed from whitish to a bright orange color. The expelled pycnospores were described as lemon yellow, later becoming a chestnut brown. In 1912 [2] and 1913 [3] Clinton described cultures of *E. gyrosa*, which is the *E. radicalis* of the present writers and will be so named hereafter in this paper, on potato, Lima-bean, and oat agars. The following differences between this species and *E. parasitica* were pointed out: *E. parasitica* fruited earlier and more abundantly than *E. radicalis* and had more numerous and smaller fruiting bodies than the latter; *E. parasitica* also produced a less luxuriant aerial growth, the embedded mycelium being much more highly colored. In his last paper, 1913,[3] Clinton gives further accounts of cultures, emphasizing the points already mentioned and giving five differences in the behavior of the two. The principal point, in addition to those just mentioned, is that *E. parasitica* produces more abundant, earlier, smaller pycnidia, which are embedded in the surface, while *E. radicalis* forms fewer, elevated, distinct pustules, rarely hidden by the exuding spores. Cultures on potato-juice agar, with various quantities of tannic acid, were also compared. Clinton found that *E. parasitica* grew in higher percentages of the tannic acid used and produced a more evident development of mycelium than *E. radicalis*.

The Andersons [4] pointed out the differences between cultures of *Endothia parasitica* and *E. radicalis* (*E. virginiana*) grown on potato agar and on sterile chestnut twigs. They found practically the same differences between the two organisms on the potato agar as described by Clinton, though somewhat more detail is given in regard to the development of the characteristic features. They also pointed out, for the first time, the differences in the growth of the two species on sterile chestnut twigs. *E. parasitica* produced a short, white, weblike growth on the surface of the twigs, with heavier masses of mycelium, which later became orange colored where pycnidia developed, while *E. radicalis* developed a fluffy orange

[1] Clinton, G. P. Chestnut-bark disease, Diaporthe parasitica Murr. Connecticut Agricultural Experiment Station, 31st–32d Annual Reports, [1906]–1908, p. 879–890, 1908.

[2] Clinton, G. P. The relationships of the chestnut-blight fungus. Science, n. s., v. 36, no. 939, p. 907–914, 1912.

[3] Clinton, G. P. Chestnut-bark disease. Connecticut Agricultural Experiment Station, Annual Report, 1912, pt. 5, p. 422, 1913.

[4] Anderson, P. J., and Anderson, H. W. The chestnut-blight fungus and a related saprophyte. Phytopathology, v. 2, no. 5, p. 204–210, 1912.

mycelial growth almost filling the tube, at first white, but changing to orange within a few days.

Pantanelli [1] describes cultures of both species on glucose agar to which extract of chestnut bark had been added. He says the appearance and quantity of mycelium was the same in both, but *Endothia radicalis* developed a characteristic "giallo-aranciato" yellow-orange color less rapidly than *E. parasitica*. He also grew both on sterile chestnut branches and roots. On the roots *E. radicalis* grew slowly and *E. parasitica* much less or not at all.

Fulton [2] mentions cultures on artificial media, stating that *Endothia parasitica* grows most readily on an acid medium.

Heald and Gardner [3] found that cultures from pycnospores and ascospores of *E. parasitica* could be distinguished by the earlier appearance of the growth from ascospores in plates. A 3 per cent dextrose agar +10 was used.

CULTURE MEDIA.

The following are the principal media and substrata upon which the organisms have been grown: Sterile chestnut twigs, Raulin's fluid, potato cylinders, chestnut-twig agar, beef agar −15 Fuller's scale, prune agar +11 to −15, corn-meal agar +11 to −15, potato agar +15 to −15; also starch, oatmeal, rice, and white corn meal in flasks. The stock cultures from which subcultures were made were kept on corn meal in flasks. The subcultures, unless otherwise stated, were made by transferring pycnospores from the stock cultures. The cultures were kept in dark cases at ordinary room temperatures, except where otherwise mentioned.

LIGHT TESTS.

Two series of spore streaks of *Endothia parasitica* and *E. radicalis* on potato agar +6 were placed in a beaker in bright light on a window sill. Duplicate series were wrapped in heavy opaque paper projecting well above but not covering the plugs.

After two weeks *Endothia parasitica* showed slightly more orange color in the darkened tubes. This difference was probably due, however, to the fact that evaporation was less in the dark tubes rather than to the want of light.

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[1] Pantanelli, E. Su la supposta origine europea del cancro americano del castagno. Atti, Reale Accademia dei Lincei, ser. 5, Rendiconti, Classe de Scienze Fisiche, Matematiche e Naturali, v. 21, fasc. 12, p. 869-875, 1912.

[2] Fulton, H. R. Recent notes on the chestnut bark disease. Conference Called by the Governor of Pennsylvania to Consider Ways and Means for Preventing the Spread of the Chestnut Tree Bark Disease. Harrisburg, p. 48–56, 1912.

[3] Heald, F. D., and Gardner, M. W. Science, n. s., v. 37, no. 963, p. 916–917, 1913.

MOISTURE TESTS.

Three series of several flasks each on corn meal were made with different quantities of water, using *Endothia radicalis* and *E. parasitica*, as follows:

Series 1.—Corn meal, 10 grams, plus water, 10 cubic centimeters.
Series 2.—Corn meal, 10 grams, plus water, 15 cubic centimeters.
Series 3.—Corn meal, 10 grams, plus water, 20 cubic centimeters.

The two species grew equally well in each series. There was, however, a marked difference as to the time of appearance of spores in the different series. Spore production began at about the same time with both species in the same series.

At the end of two weeks in series 1, *Endothia parasitica* showed numerous small spore pustules. *E. radicalis* at the same age showed but four pustules in each flask. These pustules were much larger than those of *E. parasitica*. In series 2 both species showed an intermediate condition of development. In series 3 *E.parasitica* developed somewhat more mycelium than in series 1, and there were only a few scattered spore pustules. *E. radicalis* produced more mycelium than in series 1 and only two or three very small pustules.

At the end of two months in series 1 *Endothia parasitica* had covered the entire surface with small confluent spore masses. *E. radicalis* now showed numerous large pustules with extruding spore masses. Series 2 was intermediate, as before. In series 3 *E. parasitica* showed a large number of separate small spore pustules with white mycelium between them, while *E. radicalis* showed but few stromata, much enlarged and extruding large spore masses.

It will be noted that the greatest spore production occurred in the cultures having the least quantity of water added.

TEMPERATURE TESTS.

Fulton [1] found that conidia germinate best at a temperature of 60° F. (15.5° C.) and distinctly less rapidly at 10 degrees above or below the optimum. Ascospores germinate best at a temperature of about 70° F., but give a good percentage of germination at 85° and 45° F. He also found that early growth is most rapid at the optimum temperature for germination.

A few experiments have been made by the writers to determine the temperature relations of the organisms.

Series 1 (six tubes of each) on potato agar+7.—At the end of 14 days the cultures kept at 40° C. showed no growth in any case.

At 35° C. *Endothia parasitica* showed a small amount of an irregular rough leathery growth, orange next to the agar. *E. radicalis* showed

[1] Fulton, H. R. Op. cit.

only a slight amount of growth in a few tubes. *E. gyrosa* showed about the same amount of growth as *E. parasitica*, but the growth was decidedly more normal in appearance. Only two of the tubes of *E. radicalis* which had shown no growth at 35° C. began to grow when kept at room temperature.

In one week at room temperature, 22° to 24° C., *Endothia parasitica* and *E. gyrosa* nearly, if not quite, covered the surface of the medium, while *E. radicalis* showed but little less growth. In 14 days all species at room temperature completely covered the surface of the medium with a typical mycelium. This appears to be at least nearer the optimum temperature than any other used.

At 18° C. growth in all species was much less than at room temperature. There was, however, a marked and typical difference between *Endothia parasitica* and *E. radicalis*, which was more pronounced than is usually the case at room temperature. The differences were practically the same as those described for potato-agar cultures on page 11.

At 16° C. the cultures differed very little from those at 18° C.

At 7° C. there was no growth whatever.

Series 2 (six tubes of each) on potato agar +6.—At the end of 15 days cultures at 40° C. showed no growth.

At 35° C. *Endothia parasitica* showed a slight abnormal growth, as described in series 1. *E. radicalis* showed no growth whatever. *E. gyrosa* showed slightly more than *E. parasitica* and was decidedly more normal in appearance. Transferred to room temperature about half of the *E. radicalis* cultures grew.

Series 3 on corn-meal and potato agars.—In this test there was no noticeable difference in the behavior of the fungi on these two media. Cultures from over 70 different specimens chosen from the extremes of the known ranges of the several species and from near the center were used. These cultures were made partly from spores and partly from mycelium. In no case was any difference noted in the cultures of the same species from the various localities or different inoculating material. At the end of 24 days there was no growth in any at 40° C.

At 35° C. *Endothia parasitica* showed an abnormal growth, as described for series 1. Material from New Hampshire and from Virginia showed no difference. *E. radicalis* showed no growth. *E. gyrosa* showed growth about equal to that of *E. parasitica*. Cultures from Michigan and Alabama showed no difference in the amount of growth.

At 32° C. *Endothia parasitica* produced a fairly abundant growth, which had taken on the characteristic orange color. *E. radicalis* showed but a slight amount of growth. *E. gyrosa* showed a little more development than *E. parasitica*.

At 30° C. there was less growth in all cases than at room temperature. *E. parasitica* showed slightly less orange than at room temperature. At room temperature the growth was typical, as described for series 1. At 16° C. the growth in all was almost exactly as described for 18° C., series 1. At 12° C. all were typical in character, but less in amount than at 16° C. At 9° C. there was very slight growth, about uniform in all. At 7° C. there was no growth.

From these records it will be noted that the minimum temperature for all was 9° C. and that all failed to grow at 7° C. The maximum temperature for *Endothia parasitica* and *E. gyrosa* was 35° C. The maximum for *E. radicalis* was 32° C. At higher temperatures there was no growth. Thus, there is apparently a difference of 3 degrees C. between the maxima of *E. parasitica* and *E. radicalis*. What practical or biological significance this fact may have is not clear to the writers. Owing to a lack of facilities for controlling temperatures between 18° C. and 28° C. the optimum for the organisms could not be accurately determined. The optimum appears to be near ordinary room temperature, i. e., about 22° to 24° C.

DESCRIPTION OF CULTURES.

In the following descriptions of cultures all names of colors are taken from Ridgway's [1] recent work on color nomenclature.

No growth whatever was obtained on Raulin's fluid, beef agar −15, corn-meal agar +11, and prune agar +11. In the two last-named cases the media were so acid that they would not solidify.

Potato cylinders.—On ordinary potato cylinders sterilized in an autoclave at 115° C. all the organisms grew satisfactorily, but no differential characters of value appeared before the cultures dried up.

Chestnut twigs.—Cultures on sterilized chestnut twigs showed the same characteristics of growth for *Endothia parasitica* and *E. radicalis* as described by the Andersons. These species are most easily distinguished on the cut surfaces of the twigs.

Endothia radicalis mississippiensis showed at the end of six weeks a cadmium-orange mycelium on the cut surfaces of the twigs, while *E. radicalis* and *E. parasitica* were cadmium yellow in the colored portions.

Endothia gyrosa showed at first an ochraceous-buff growth on the cut surfaces and later changed to nearly the same color as *E. radicalis*. *E. gyrosa* may, however, even in old cultures, be distinguished from the others by the irregular tubercles or subpulvinate masses of mycelium.

[1] Ridgway, Robert. Color Standards and Color Nomenclature. Washington, 43 p., 53 pl., 1912.

CULTURES ON CHESTNUT-TWIG AGAR.

The chestnut-twig agar was prepared by the following formula:

To 275 grams of 1 or 2 year old chestnut branches add 550 cubic centimeters of distilled water and boil over an open flame for one-half hour. Filter the juice and make up to 550 cubic centimeters with distilled water. To 50 parts of this infusion add 100 parts of distilled water and 2 per cent of agar flour. Steam for one-half hour, filter, tube, and autoclave for 15 minutes at 115° C.

Several different strengths of the infusion were tried, but the formula given above proved the most satisfactory. The series of cultures on this medium gave readily distinguishable differences between *Endothia parasitica* and *E. radicalis*, but growth was too slow for general use. After one month *E. parasitica* showed numerous spore masses and an abundant white surface growth, but no aerial hyphæ. *E. radicalis* showed no spore production and had considerable white, fluffy, aerial mycelium at the upper edge of the agar. At the end of two months a few of the tubes of *E. radicalis* showed one or two spore pustules, while the others were unchanged.

CULTURES ON PRUNE AGAR.

Prune agar was prepared according to the following formula:

Take four ordinary prunes and add 1 liter of water. Boil over an open flame for one hour, being careful not to break the skin of the prunes. Strain through gauze, make up to the original amount with distilled water, and add 2 per cent of agar flour. Steam for three-quarters of an hour, filter, and tube. Autoclave for 15 minutes at 115° C.

Cultures of *Endothia parasitica* 3 weeks old on this medium, −15 Fuller's scale, produced a great abundance of pycnidia with slimy spore masses exuding, but no aerial mycelium. *E. radicalis* from both American and European material produced few small pycnidia and spore masses but somewhat more aerial mycelium, especially at the bottom of the tubes. The other species were not grown on this medium.

CULTURES ON CORN-MEAL AGAR.

Formula: To 50 grams of corn meal add 1 liter of water. Keep in a water bath for 1 hour at a temperature of 58° C., never over 60° C. Filter through paper, add $1\frac{1}{4}$ per cent of agar flour, steam for $1\frac{1}{2}$ hours, filter, and tube. Autoclave for 15 minutes at 115° C.

Corn-meal agar made by the above formula generally tests +3. This has proved the best agar medium for the production of pycnospores. There is also a constant difference in the cultural characters of the different species on this medium. This difference is, however,

much more striking on unslanted tubes. Upright tubes have the disadvantage of a small surface, but this is more than offset by the fact that the greater depth of agar gives a much more uniform moisture supply to the fungus. The most characteristic differences upon this medium appear in cultures from 6 weeks to 2 months old. The earlier stages are also sufficiently distinct for easy identification. In the older stages *Endothia parasitica* showed a scant white growth of surface mycelium with several prominent pycnidial pustules clustered near the center and of a slightly darker shade than the raw sienna of Ridgway. *E. radicalis*, on the other hand, produced a rather abundant deep chrome mycelium with usually one or two smaller, less conspicuous pycnidial pustules. *E. radicalis mississippiensis* on this medium resembled *E. parasitica* in producing only a very scant surface growth of mycelium, but the color was between cadmium yellow and raw sienna. The pycnidial pustules were more numerous than in *E. radicalis* and smaller and more scattered than in *E. parasitica*.

At this age *Endothia gyrosa* showed rather abundant, felty white mycelium flecked with capucine buff but no pycnidia. Occasionally in old cultures of this species small pycnospore threads were produced. On corn-meal agar −15 the growth was slow, but an abundance of spores was produced.

CULTURES ON POTATO AGAR.

The formula for the potato agar used is as follows:

Put clean pared potatoes through a meat grinder. To 1,000 grams of the potato pulp add an equal quantity of distilled water. Stir thoroughly and let stand in an ice box for an hour, with occasional stirring. Strain through gauze of medium mesh. Make up to three times the weight of the original pulp with distilled water. Steam for 1 hour, filter through cotton and paper, and make up to 3,000 cubic centimeters with distilled water. Add 1½ per cent of agar flour, steam for 1 hour, filter through cotton and paper, tube, and autoclave for 15 minutes at 115° C.

In practice it was found that the potato agar prepared according to this formula varies widely in acidity and probably in some other respects. To reduce this variation a large quantity of potato juice was made from a uniform lot of Burbank potatoes. This was placed in 1,000-cubic-centimeter flasks, tightly plugged and kept in a refrigerator. The juice was then made up into agar tubes as needed. It was found that this agar varied less than 1 per cent in acidity, changing from +7 to +6 during 5 months.

Endothia parasitica.—Pycnospore streaks of *Endothia parasitica* and *E. radicalis* on this potato agar agreed closely with that described

by Anderson. At the end of 3 or 4 days *E. parasitica* at room temperature showed a short, fluffy, white, aerial growth along the streak. The surface of the mycelium was orange by transmitted light, while by reflected light it was between raw sienna and antique brown at the sides. Within 6 days the mycelium, especially at the base of the agar slant, took on a peculiar granular metallic appearance, due apparently, in part at least, to the character of the mycelium and in part to the minute water drops scattered over the surface. This portion of the culture was light orange yellow by reflected light and orange by transmitted light. The peculiar surface appearance might perhaps be called "brassy." This metallic appearance has been found to be the most constant and reliable distinguishing character of *E. parasitica* on potato agar. In 12 to 14 days small pycnidial pustules appeared in the upper portion of the tubes, and the agar just below the mycelium became warbler green, changing later to olive green.

Endothia radicalis.—Pycnospore streak cultures of this species vary widely as to the amount and time of the appearance of color. Occasionally tubes showed a small orange area in 5 days. Many tubes showed the same color in 1 week, while others produced no orange color whatever. In no case did cultures of *Endothia radicalis* produce the brassy metallic surface so characteristic of *E. parasitica.* Pycnidia were few and more scattered than in *E. parasitica* and spore masses did not begin to appear until the third or fourth week. A slight amount of warbler-green color appeared in the medium occasionally at this age, but never so conspicuously as in *E. parasitica.*

Cultures made by transfer of mycelium of *Endothia radicalis* to potato agar were found to vary widely as to the rate of growth, color, and general appearance. The variations in such cultures were so great as to make them much less reliable than spore cultures.

Endothia radicalis mississippiensis.—This produced less fluffy aerial mycelium along the spore streak than *Endothia parasitica.* After 5 or 6 days the fungus showed an orange color by transmitted light and was indistinguishable in this respect from *E. parasitica.* The character of the surface was somewhat different, however, and by reflected light appeared xanthine orange. When 2 weeks old this form differed still more markedly from *E. parasitica* in color, being grenadine red by transmitted light and showing no spore masses.

Endothia gyrosa.—This species developed more slowly than any of the others, producing a rather abundant aerial growth, which was felty rather than fluffy. The color was white flecked with capucine buff, and no spore masses were produced.

On potato agar of high acidity, i. e., from +11 to +15, no spores were produced in any of the species, even in very old cultures. In agar of lower acidity, spore production was proportionately greater,

as well as earlier in appearance. The high-acid media, however, gave the distinctive colorations described more quickly than those of lower acidity.

CULTURES ON OATMEAL.

The oatmeal is prepared as follows: Put 10 grams of oatmeal in 100-cubic-centimeter Erlenmeyer flasks. Add 20 cubic centimeters of distilled water. Stir until thoroughly mixed, plug, and autoclave for 25 minutes at 120° C.

Starch, rice, and white corn meal are prepared in the same manner, but cultures of all species developed so slowly on starch in flasks that this medium was abandoned. Corn meal in 1-inch test tubes prepared in the same manner was tested with 10 strains of *Endothia parasitica* and 30 of *E. radicalis*, using about an equal number of American and European strains. This did not show the same color reactions as in the flasks. Only about half of the tubes of *E. radicalis* showed purple before the medium dried up.

Endothia parasitica.—Cultures 1 week old showed a superficial, more or less erect, pure white cottony growth covering the whole surface of the medium. *Endothia parasitica* on the same medium 12 days old began to show spore masses extruding through the white mycelium near the point of inoculation. At the end of a month the spore masses, which were pale yellow ocher, were rather numerous, of medium size, and scattered over the surface of the culture. The older portions of the mycelium in contact with the glass were orange. At 6 weeks of age cultures showed more or less sunken patches very near flame scarlet, whereas cultures on other media of the same age showed no change.

Endothia radicalis.—Cultures at the end of a week showed a growth about as luxuriant as in *Endothia parasitica*, but the aerial portion had a slightly coarser and more tufted appearance. The color ranged from light orange yellow through perilla purple to light pinkish lilac at the margin. The older portion of the culture in contact with the glass was perilla purple, apparently in both mycelium and medium. On the same medium at the end of a month there was a somewhat greater development of mycelium, with a more compact surface and irregular small cushionlike masses. The perilla-purple color had almost entirely disappeared from the surface, but could be seen through the oatmeal on the bottom and sides of the flask. The mycelium in contact with the flask was orange. No spore masses were present.

Endothia radicalis mississippiensis.—Cultures 1 week old showed a buff growth covering the surface of the medium. About three-fourths of the older portions were orange chrome. The superficial

growth was very similar in character to that of *Endothia parasitica*, especially the pure white portion near the margin. The older portions appeared slightly coarser and tufted. Cultures on the same medium 1 month old showed a still coarser aerial growth, with the very youngest portions nearly white, but the greater part of the growth was orange rufous. This color was also present wherever the mycelium came in contact with the flask. The culture medium showed no change in color. Spore masses appeared in these cultures in about the same time as in *E. parasitica*. They were scattered and inconspicuous on account of being practically the same color as the older portions of the mycelium, and were practically the same size and character as in *E. parasitica*.

Endothia gyrosa.—Cultures made from mycelium, no pycnospores being available, showed a superficial growth covering the surface of the medium. The mycelium was of a softer and more cottony appearance than in the other species, and pure white except a small portion at the point of inoculation, which was ochraceous buff. Cultures a month old showed a more compact surface, which was rather pulvinate or pustulate. The spore masses were of the same ochraceous-buff color, and the other portions lighter. The margin in contact with the flask was dark brown.

CULTURES ON RICE.

Endothia parasitica.—In cultures 1 week old the growth covered about one-fourth of the surface of the medium with a scanty superficial mycelium. The mycelium was white, flecked with buff yellow, the color being more conspicuous at the margin. The color of the medium was unchanged. No spore masses were found.

The same fungus in cultures 1 month old showed the surface growth unchanged in character, but dirty white with numerous small spore masses which were somewhat confluent near the center of the growth and showing a pale yellow ocher color. The medium was mostly tea green on the bottom, streaked with lighter green, giving it a marbled appearance. The mycelium on the bottom of the flask was about the same color as the spore masses.

Endothia radicalis.—Cultures 1 week old showed a loose, fluffy, erect mycelium covering nearly the whole surface of the mycelium. This was deep chrome to light orange yellow at the point of inoculation, passing through perilla purple and light pinkish lilac and fading into white at the margin. The mycelium had penetrated to the bottom of the flask, producing a sort of mosaic of colors. Where the rice grains came in contact with the glass they were perilla purple, and in the interstices the mycelium varied from apricot yellow to white. No spore masses were present.

The same species 1 month old gave a surface growth rather thick and abundant, much more compact than in the younger cultures, and ranging from buff yellow to white. The medium in contact with the glass was marbled, the deepest color being corinthian purple and the lightest ecru drab.

Endothia radicalis mississippiensis.—In cultures 1 week old the mycelium covered the whole surface of the medium. The aerial growth was erect, fluffy, much less in quantity than in *Endothia radicalis* and very similar to *E. parasitica* in character. The color was the same as in *E. parasitica* but more extensive. The medium was unchanged in appearance and no spore masses were seen.

The same fungus at the age of 1 month showed a thick, rather compact growth, somewhat more dense than in *Endothia radicalis* of the same age. The color of the mycelium on the surface was cadmium orange, changing to xanthine orange where in contact with the glass. The spore masses were minute, rather numerous, and of a pale yellow ocher color. The medium at the bottom first showed perilla purple when the cultures were 20 days old.

Endothia gyrosa.—Cultures from mycelium when 1 week old covered the whole surface of the medium. The growth was very thin, loose, and fluffy, pure white except at the point of inoculation and where in contact with the glass. At these points it was a light ochraceous buff with a faint suggestion of purplish lilac. The color of the medium was unchanged and no spores were produced.

The same fungus when 1 month old showed an abundant thick growth with a cottony tomentose pulvinate surface. The surface mycelium was capucine yellow, changing to xanthine orange where in contact with the glass. The color of the medium was marbled, ranging from lumiere green to apple green. The line marking the boundary between the mycelium and the culture medium on the sides of the flask was sooty black. No spores were found.

CULTURES ON CORN MEAL.

Endothia parasitica.—In cultures 1 week old the growth on corn meal covered about one-half of the surface of the medium. The outer margin was pure white, the remainder buff yellow below, with a superficial white growth above and the medium uncolored. A few small pustules with spore masses occurred near the point of inoculation.

Cultures 1 month old showed a compact growth nearly smooth on the surface. The superficial mycelium was pale orange yellow. The pale yellow ocher spore masses were minute, very numerous, and nearly covered the surface. The medium was slightly greenish about the sides of the flask just beneath the mycelium.

Endothia radicalis.—Cultures at the age of 1 week showed a growth of loose, fluffy mycelium covering one-half the surface of the medium. The growth was less in quantity and also less compact than that on rice and oatmeal. The same colors were present as in the cultures on rice, but paler. No spores were present.

Cultures 1 month old showed a compact growth with a nearly smooth surface. The color ranged from light cadmium to empire yellow. The whole mass of the medium was perilla purple. No spore masses were found.

Endothia radicalis mississippiensis.—Cultures 1 week old showed a growth very similar to that on oatmeal, but less in extent and in aerial development. The color of the mycelium was orange chrome. There was no discoloration of the medium, and no spore masses were found.

The same form of the fungus from the same source 1 month old produced a growth with a compact, rather uniform surface, the superficial portion having a coarse, matted, webby appearance, which was most noticeable about the margin. The color of the mycelium was cadmium orange to xanthine orange, while that of the medium was unchanged. Spore masses were much more numerous than in *Endothia radicalis*, but smaller and less numerous, though very similar to those of *E. parasitica*.

Endothia gyrosa.—Mycelial cultures 1 week old showed a growth practically the same in quantity as in *Endothia radicalis* but more compact on the surface and ochraceous buff near the point of inoculation, shading into white at the margin. There was no discoloration of the medium, and no spore masses were seen.

Cultures of the same kind 1 month old showed an abundant, rather thick growth, having the surface mostly covered with somewhat irregular tubercular masses of mycelium, suggesting immature pycnidial stromata similar to those formed in *Endothia radicalis*, but smaller and producing no spores. The surface of the culture was capucine buff. The surface of the tubercles was honey yellow to Isabella. The dark color was apparently due in part to numerous superficial water drops. A portion of the medium was perilla purple.

Aside from the differences in color, the most conspicuous and important characteristic of these fungi in corn-meal cultures is found in the fructification. Clinton has already mentioned and illustrated similar differences in cultures of these organisms on agar in Petri dishes. In *Endothia parasitica* the pycnidia and spore masses are small, numerous, thickly scattered, and embedded in the mycelium. *E. radicalis*, on the other hand, forms but few large, erumpent stro-

mata, with spores extruding in thick, elongated masses. *E. radicalis mississippiensis* appears somewhat intermediate between *E. parasitica* and *E. radicalis* in regard to the character and abundance of the pycnidia and in color of the growth. These peculiarities have been very uniform and constant in all the cultures on this medium, and if they could be coordinated with regular morphological differences in nature would justify its separation as a species.

SUMMARY.

The chestnut-blight fungus *Endothia parasitica* has three rather close relatives, *E. radicalis* (*E. virginiana* And. and And.), *E. radicalis mississippiensis*, and *E. gyrosa* (*E. radicalis* of Clinton), which in their pycnidial condition are not readily distinguished with certainty by either macroscopic or microscopic examination.

Physiological studies of these organisms in pure cultures have been made to test their relation to light, moisture, and temperature, and also their behavior on different culture media. All behaved the same in light and darkness, growing about equally well in either.

Cultures of *Endothia parasitica* and *E. radicalis* were made on corn meal to which different quantities of water had been added. The cultures with equal quantities of corn meal and water produced pycnidia and spores more abundantly than those to which the greater quantities of water were added.

Temperature tests showed that 8° to 9° C. is the least temperature at which any of the three species will grow. The highest temperature at which growth occurred was 35° C. for *Endothia parasitica* and *E. gyrosa*, and 32° C. for *E. radicalis*. The best temperature for growth was between 18° and 28° C. for all.

Studies of over 2,000 pure cultures of these fungi appear to show that they possess constant and easily recognized cultural characters on several culture media, of which potato agar and corn meal are the best that have been tried.

The most distinctive and reliable cultural character of *Endothia parasitica* is the peculiar, granular, glistening light orange-colored surface growth produced at the bottom of potato-agar slants. This usually appears within a week. None of the other organisms have developed this character, though *E. radicalis* occasionally in very old cultures shows suggestions of it. The orange color of the mycelium of *E. parasitica* as seen by transmitted light usually appears in three or four days, and this is ordinarily sufficient for identification. In the other species this color is not produced at all, or in case of *E. radicalis* not until a few days later.

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On corn meal *Endothia parasitica* was constantly characterized by the early production of numerous small pycnidia and the want of any distinct color changes in the medium, while in *E. radicalis* the pycnidia are few and large, appearing later, and the medium becomes perilla purple. In *E. radicalis mississippiensis* the pycnidia are larger and less numerous than in *E. parasitica* and the color of the mycelium is orange chrome at the end of a week. *E. gyrosa* is distinguished from *E. parasitica* by its very tardy spore production and the formation of elevated or subcolumnar pycnidial stromata.

[Cir. 131—B]

MEASURING HAY IN RICKS OR STACKS.[1]

By H. B. Mc Clure, *Agriculturist*, W. J. Spillman, *Agriculturist in Charge*, and J. W. Froley, *Agriculturist, Office of Farm Management.*

INTRODUCTION.

The methods presented here for determining the number of tons of hay in a rick or stack are in no way official. They simply represent the results of a somewhat extended investigation of the subject covering a period of several years. It is believed, however, that the method here presented for determining the number of cubic feet in a rick or stack of hay is quite accurate. The formulas given should be considered merely as the nearest approach we are able to make with our present knowledge.

THE PROBLEM OF MEASURING HAYRICKS.

The problem of obtaining an accurate formula for determining the number of cubic feet in a hayrick is difficult because of the variation in the shape of ricks. Even if the number of cubic feet in a rick is known, the problem of converting this into tons is difficult because of the wide variation in the compactness of hay in bulk.

The study of this subject was begun in the old Office of Grass and Forage-Plant Investigations, from which the Office of Farm Management developed. The actual number of cubic feet in a ton of hay in ricks has been measured in 92 cases. The results are very variable and show that any general rule for determining the weight of hay in a rick by measurement must be only an approximation. But thousands of tons of hay are sold annually on such measurements, and therefore it is desirable to establish at least average values for the quantities concerned in these measurements.

MEASURING THE VOLUME OF A HAYRICK.

The volume of a rick is equal to its length multiplied by its cross section. The length is easily measured. Let us consider the principles involved in measuring the area of the cross section. If the top of the rick were perfectly flat and the two sides straight up and

[1] Issued July 5, 1913.

down, the area of the cross section would simply be the width multiplied by the height. If the rick were triangular in cross section, so that the sides represented straight lines from the top of the rick to the bottom on each side, the area of the cross section would be one-half of the product of the base and the height. The actual area of the cross section lies somewhere between these two.

It is difficult to measure accurately the height of a rick. It is much easier to measure the "over," which is the distance from the ground on one side of the rick over the top of the rick to the ground on the other side. The length of the over depends upon three things: (1) Width, (2) height, and (3) "fullness" of the rick. The over is always somewhat more than twice the height.

It has been found by actual measurement that the cross section of a rick is the product of the over and the width, multiplied by a fraction varying from 0.25 to 0.37 (average value, 0.31), depending upon the height and fullness of the rick. If the rick is low in comparison with its width and nearly triangular in outline—that is, its sides are not very full—the fraction is small (0.25). If the rick is tall in comparison with its width, and the sides are very full, so that the top is well rounded, the fraction is large (0.37). Representing this fraction by F, the over by O, the width by W, and the length of the rick by L, the volume being represented by V, we have the following formula for determining the number of cubic feet in a rick:

$$V = F\,O\,W\,L.$$

The fact that the right-hand member of this formula spells the word "fowl" makes it easy to remember.

Figure 1 shows the cross sections of hayricks of nine different shapes, the corresponding value of the fraction F for each of these shapes being inserted in the outline of each cross section. The height of ricks Nos. 1, 4, and 7 (upper row) is three-fourths the width. The height of ricks Nos. 2, 5, and 8 (middle row) is equal to the width, while the height of ricks Nos. 3, 6, and 9 (lower row) is one and one-fourth times the width. Ricks Nos. 1, 2, and 3 (left column) are narrow or nearly triangular in outline; ricks Nos. 4, 5, and 6 (middle column) are medium full, while ricks Nos. 7, 8, and 9 (right column) are full and rounded. It will be noticed that the value of F is the same (0.31) in Nos. 3, 5, and 7; in Nos. 2 and 4 it is 0.28; and in Nos. 6 and 8, 0.34.

In attempting to find the volume of hayricks the choice between these various values of F may be found by comparing the shape of the end of the rick—that is, the cross section of the rick—with ricks Nos. 1 to 9 in figure 1. If the shape of the rick to be measured is intermediate between those shown in figure 1, intermediate values

of F may be used. The use of the above formula may be made clear by a few examples:

Example 1.—A hayrick is 16 feet wide, 24 feet long, and the over is 31.2 feet. The end view indicates that the shape of the rick is very close to No. 4 in figure 1. What is the volume of the rick?

Solution: $V=0.28\times31.2\times16\times24=3,354.6$ cubic feet.

Example 2.—A hayrick is 14 feet wide, 20 feet long, and the over is 34.2 feet. Inspection of the end of the rick shows that it is of the type of No. 8 in figure 1. What is the volume of the rick?

Solution: $V=0.34\times34.2\times14\times20=3,255.8$ cubic feet.

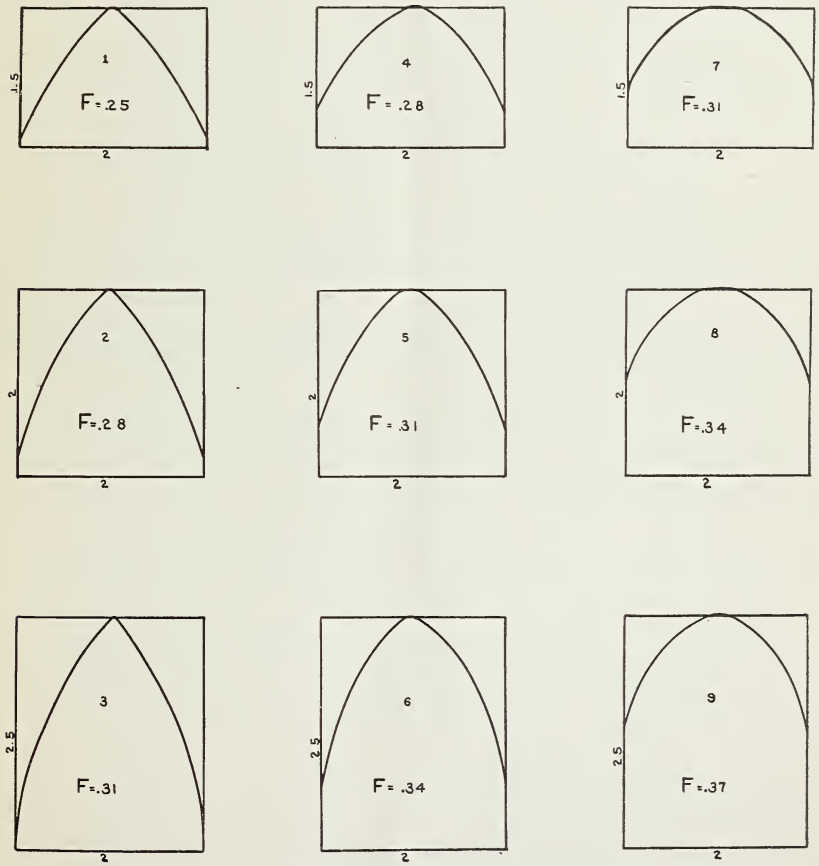

FIG. 1.—Cross sections of hayricks of different shapes.

MEASURING ROUND HAYSTACKS.

The measurement of the number of cubic feet in a round stack of hay is more difficult than it is for a rick. Simple formulas for this can be developed, however, by considering the round stack in two parts. The lower part is usually cylindrical, or in some cases drawn in at the bottom so as to present in outline the appearance

of a large pan, narrower at the bottom than at the top. The upper part varies in outline from a cone at one extreme to half a sphere at the other. The upper row of outlines in figure 2 represents these two extreme forms of top and a form intermediate between them. The lower row of outlines in figure 2 represents the two common shapes of the bottom part of round stacks. It is necessary to calculate the volume of the top and bottom parts separately and then add them together.

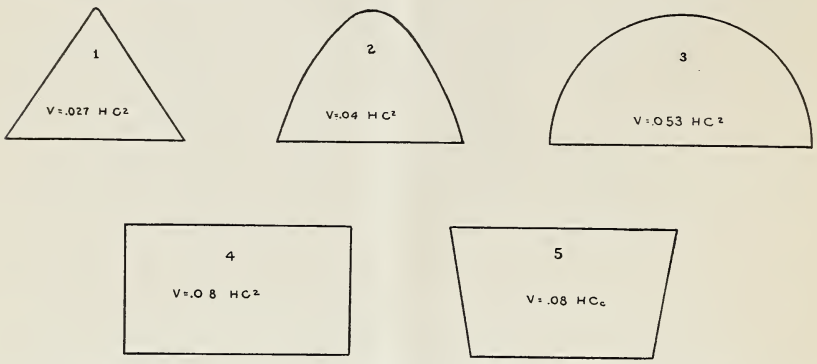

FIG. 2.—Diagram showing various shapes of round haystacks: 1, 2, and 3, Upper part of stacks (above the bulge); 4 and 5, lower part of stacks (below the bulge). Formulas for calculating the volume are given in each figure.

Formulas [1] for making these calculations are given on the outline drawings in figure 2. Thus, in drawing No. 1, the formula for finding the volume of a perfectly conical top is—

$$V=0.027\ HC^2.$$

In these formulas H represents the height of that portion of the stack being measured (not the full height of the stack), while C represents the circumference of the bottom portion of the top—that is, the circumference at the bulge or shoulder of the stack—and the small superior figure "2" to the right of C indicates that this circumference is to be squared.[2] The formulas for outlines Nos. 2, 3, and 4

[1] The method of deriving the formulas for determining the volume of round haystacks will be apparent from the following equations, $\pi=3.1416$:

Solid.	Volume.
Cone	$\frac{1}{3}\pi R^2 H=0.027\ HC^2$.
Paraboloid	$\frac{1}{2}\pi R^2 H=0.040\ HC^2$.
Spheroid	$\frac{2}{3}\pi R^3=0.053\ HC^2$.
Cylinder	$\pi R^2 H=0.080\ HC^2$.
Frustrum	$H\sqrt{\pi R^2 \pi r^2}=0.080\ HCc$.

[2] This formula is obtained as follows:

$$V=\frac{1}{2}\sqrt{\frac{1}{R}-\sqrt{\frac{1}{H^2}}};\ V=0.08\ HC^2.$$

need no further explanation. The use of these formulas will be indicated below.

The formula for drawing No. 5 in figure 2 ($V=0.08\ HCc$) means that the volume of the lower part (from the shoulder, or bulge, down) of a round stack shaped like the drawing is equal to 0.08 multiplied by the height of the base, this total multiplied by the circumference at the top of the base, and the product then multiplied by the circumference at the bottom of the stack.

In determining the volume of the tops of stacks it will be noticed that the decimal preceding the H in each of the formulas varies with the shape of the top. In order to use this formula in any given case, it will be necessary to determine by inspection what decimal to use. It will lie somewhere between 0.027 and 0.053. In measuring the volume of the base of the stack no such uncertainty exists, since the decimal in the formula is the same in all cases.

A few examples will illustrate the use of these formulas.

Example 1.—The base of a haystack is cylindrical and the top conical. The height of the base is 4 feet and the height of the top is 6 feet, the circumference of the stack at the ground and at the shoulder being 28 feet. What is the volume of the stack in cubic feet?

Solution:

Top of haystack	$V=0.027\times6\times28^2$	=127.0 cubic feet.
Bottom of haystack	$V=0.08\ \times4\times28^2$	=250.9 cubic feet.
Total		377.9 cubic feet.

Example 2.—A haystack has a top similar in shape to outline No. 2 in figure 2 and the base of the shape of outline No. 5 in figure 2. The height of the top is 5 feet, the circumference at the bulge is 26 feet, the height of the base is 3½ feet, and the circumference at the bottom of the stack is 20 feet. What is the volume of the stack in cubic feet?

Solution:

Top of haystack	$V=0.04\times5\ \times26^2$	= 95.2 cubic feet.
Bottom of haystack	$V=0.08\times3\frac{1}{2}\times26\times20$	= 89.6 cubic feet.
Total		184.8 cubic feet.

MEASURING THE HEIGHT OF HAYSTACKS.

The formula given for determining the number of cubic feet in a stack of hay involves the height of the stack, which it is necessary to measure in some manner. It is also desirable sometimes to measure the height of a rick. Figure 3 shows a simple method of accomplishing this. In using this method two points are marked on the ground on opposite sides of the rick or stack and at as nearly equal distances from its center as possible. A cord is then thrown

over the stack, stretched gently from the point A over the top of the stack to the point B and marked to show its length from A to B. The cord is then removed and placed on the ground in the position of A', B', and T', the points A' and B' being exactly the same distance apart as A and B. At T' the cord should be made to assume the shape of that portion of the top of the stack with which the cord was in contact when it was stretched over the top of the stack. The distance C' T' will then be the height of the stack.

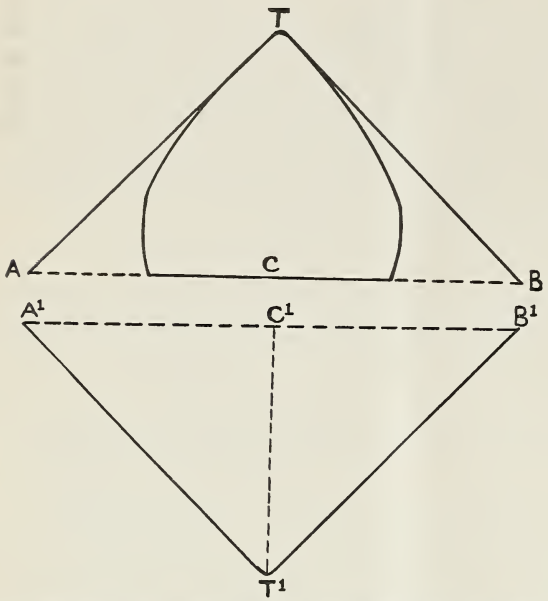

FIG. 3.—Diagram showing a method of measuring the height of stacks or ricks of hay.

NUMBER OF CUBIC FEET IN A TON OF HAY.

The following figures show the number of cubic feet in a ton of hay, based on a number of measurements, as indicated below, the hay being mainly timothy or a mixture of clover and timothy in which timothy predominated.

The average number of cubic feet per ton of hay in 55 stacks which had been standing less than 30 days was 589.6; for 30 stacks which had been standing from 30 to 60 days the average number of cubic feet per ton was 581.5, while the average for 7 stacks which had been standing from 74 to 155 days was 514.9 cubic feet per ton.

Definite measurements of other kinds of hay have not been made. The data given were obtained in the States of Virginia and New York by measuring the volume of stacks and then weighing the hay as it was baled from the stack.

[Cir. 131—C]

CELLULOSE AS A SOURCE OF ENERGY FOR NITROGEN FIXATION.[1]

By I. G. McBeth, *Physiologist, Soil-Bacteriology and Plant-Nutrition Investigations.*

INTRODUCTION.

The great chemical principle underlying nitrogen fixation by micro-organisms which was early insisted upon by Berthelot and Winogradsky is that energy in the form of organic carbon is necessary for the fixation of gaseous nitrogen by the protoplasm of the bacterial cell. Subsequent investigations have indicated that from 100 to 200 units of carbonaceous substance are utilized in the fixation of 1 unit of nitrogen; therefore, the quantity of carbonaceous material in the soil becomes an exceedingly important factor in determining its nitrogen fixing power. Within the last decade much research has been undertaken to determine the kind of carbonaceous substances best adapted to serve as a source of energy for nitrogen-fixing bacteria. These investigations have shown that a great number of compounds may be utilized either directly or indirectly by these organisms, but the nature of the compounds of greatest importance in the soil is still somewhat problematical. It seems certain, however, that the source of energy for nitrogen fixation in soils must be derived from the decomposing plant substances, and as by far the larger part of the organic carbon added to the soil as roots, stubble, etc., is in the form of cellulose, it would seem that this substance may have a far reaching effect on the development of nitrogen-fixing organisms therein.

As cellulose is an inert compound inaccessible to such nitrogen-fixing bacteria as Azotobacter it would seem that its value as a source of energy for nitrogen fixation may, in a measure at least, be dependent upon the activity of other organisms capable of breaking it down into more accessible compounds. In studying the value of these compounds as a source of energy for Azotobacter it seems desirable to grow the celluluse destroying and nitrogen-fixing organisms in association, or at least in the same nutrient solution. In such an experiment it is obvious that the solution employed must be favorable for the process of nitrogen fixation as well as for cellulose destruction.

[1] Issued July 5, 1913.

Our studies of the cellulose destroying bacteria have shown that these organisms bring about a rapid destruction of cellulose only when supplied with a considerable quantity of combined nitrogen. It therefore seemed necessary to consider first the effect of combined nitrogen in the culture media on the nitrogen fixing power of Azotobacter before undertaking to determine the value of cellulose as an indirect source of energy for nitrogen fixation by these organisms.

REVIEW OF INVESTIGATIONS ON THE EFFECT OF COMBINED NITROGEN IN THE CULTURE MEDIA ON THE NITROGEN-FIXING PROPERTIES OF MICRO-ORGANISMS.[1]

The synthetic culture media used by different investigators in the study of the nitrogen-fixing properties of micro-organisms, although showing considerable variety in composition, have usually contained little or no combined nitrogen. The absence of nitrogen in these media seems to have resulted from a very general acceptance of the early conclusions of Berthelot and Winogradsky that if an organism is grown in a medium rich in combined nitrogen there is a reduction or total loss of the nitrogen-fixing power exhibited by the organism when grown in nitrogen free media. From a review of the work of these early investigators it would seem that the results secured would hardly warrant a final acceptance of their views without additional proof.

Studies by Berthelot of nitrogen fixation with pure cultures are limited, and in these studies no special attention was given to the effect of combined nitrogen on the nitrogen-fixing power of the organisms. Berthelot believed, however, that a small quantity of combined nitrogen was required to give the organisms the minimum amount of energy necessary to enable them to utilize the free nitrogen of the air.

Winogradsky soon proved this latter conclusion to be erroneous by demonstrating the nitrogen-fixing power of an impure culture of a butyric ferment in nutrient solutions from which the last trace of nitrogen had been removed. In 13 flasks containing no nitrogen he secured a fixation of from 1.25 to 3 milligrams of nitrogen for each gram of dextrose added. When 2.1 milligrams of combined nitrogen were added to the solution, the fixation for four flasks varied from 2.1 to 3.56 milligrams for each gram of dextrose added. With three flasks in which 3, 3.3, and 4 milligrams of combined nitrogen were added the fixation was 2.5, 0.3, and 0.4 milligrams, respectively. It would seem that the addition of 2.1 milligrams of combined nitrogen had a slight stimulating effect on the nitrogen-fixing organisms, while the addition of larger quantities reduced the quantity of nitrogen fixed.

[1] For bibliography, see p. 34.

The following year Winogradsky continued his studies of the effect of combined nitrogen upon the nitrogen-fixing properties of this butyric ferment associated with two other organisms. In one series of flasks in which the nutrient solutions contained 3 grams of dextrose and quantities of nitrogen as ammonium sulphate varying from 2.1 to 21.2 milligrams the results showed the greatest gain for the flask receiving the smallest quantity of combined nitrogen, and when the combined nitrogen added amounted to 21.2 milligrams there was a loss of 2.2 milligrams. Winogradsky therefore concluded that for nitrogen fixation the ratio of nitrogen to carbohydrate should not be greater than 6 to 1,000. We should not lose sight of the fact, however, that the results secured by Winogradsky in this experiment may have been determined in a large measure by the impurity of his culture.

Work by Ashby and by Hoffmann and Hammer has indicated that a large percentage of the nitrogen fixed by impure cultures in solutions may be lost through denitrifying processes, especially if the incubation period is prolonged. The latter investigators noted a loss of 38 per cent of the nitrogen fixed in a series of flasks incubated for six weeks. In view of these results it would seem that the gains secured by Winogradsky are of somewhat doubtful value in determining the effect of combined nitrogen on the nitrogen-fixing power of micro-organisms.

In 1897 Mazé reported nitrogen-fixation studies with *Bacillus radicicola* in a decoction of white kidney bean to which 2.6 per cent of saccharose, a little sodium chlorid, and a trace of sodium bicarbonate were added. One hundred cubic centimeters of the solution contained 22.4 milligrams of initial nitrogen. However, a total gain of 23.4 milligrams of nitrogen was secured, or a gain of 9 milligrams for each gram of sugar added. The nitrogen gain reported in this rich nitrogenous medium is far in excess of gains reported by later investigators for *B. radicicola* in solutions weak in initial nitrogen.

Saida in 1901 studied the nitrogen-fixing properties of *Phoma betae*, *Mucor stolonifer*, and *Aspergillus niger* in nitrogen-free and nitrogen-containing solutions. The nitrogen fixation by these organisms was usually stimulated by the addition of small quantities of combined nitrogen.

Lipman in 1903 studied the nitrogen-fixing properties of *Azotobacter vinelandii* in mannite solutions to which small quantities of nitrogen as potassium nitrate, ammonium chlorid, and peptone were added. The gains secured did not show any appreciable effect from any of the nitrogen compounds employed. However, the quantities of nitrogen added were extremely small, amounting to less than 1 milligram to each 100 cubic centimeters of solution.

In 1904 Moore in nitrogen-fixation studies with *Bacillus radicicola* in nutrient solutions secured a fixation of 0.2 to 2.2 milligrams for each 100 cubic centimeters of nitrogen-free solution. When 0.01 per cent of ammonium phosphate was added the gains sometimes amounted to 3.1 milligrams to each 100 cubic centimeters of solution.

Stoklasa in 1908 secured the following results with a solution containing 2 per cent of mannite and 0.2 per cent of sodium nitrate held under aerobic conditions at 20° C. and inoculated with *Azotobacter chroococcum:*

Nitrogen in form of—	Milligrams of nitrogen in inoculated flasks.			Milligrams of nitrate nitrogen in sterile flasks.
	10 days.	20 days.	30 days.	
Ammonia	14.8	24.3	28.8	326.2
Nitrite	222.6	243.5	242.1	336.2
Nitrate	32.4	14.4	12.0	.....
Organic	64.8	60.5	65.2	.....
Total	334.6	342.7	348.1	.....

From this experiment Stoklasa concluded that Azotobacter does not assimilate free nitrogen in the presence of nitrate. It would seem, however, that there was a slight gain in nitrogen, as the inoculated flasks in every test show an increase over the average of the two sterile flasks, though the gains indicated are possibly within experimental error.

Bacillus radiobacter under similar conditions was found to be a violent denitrifier, liberating in 10 days 76.3 per cent of the nitrate added.

Bredemann in 1909 in studying the nitrogen-fixing properties of *Clostridium pasteurianum* and *Bacillus amylobacter* in peptone-dextrose solutions secured the following results:

Organism.	Quantity of solution employed.	Peptone nitrogen added.	Nitrogen gained.	Nitrogen gained per gram of dextrose employed.
	Cubic centimeters.	*Milligrams.*	*Milligrams.*	*Milligrams.*
Clostridium pasteurianum	500	1.600	20.656	2.07
		7.983	18.423	1.84
		39.915	17.031	1.70
Bacillus amylobacter	500	1.600	22.656	6.02
		7.983	14.583	3.24
		39.915	16.871	4.22
		79.825	10.731	2.75

In another table Bredemann shows the nitrogen-fixing power of *Clostridium pasteurianum* in dextrose solutions to which no combined nitrogen was added as varying from 0.8 to 1.3 milligrams to each gram of dextrose consumed; *Bacillus amylobacter* in a similar solu-

tion gave 0.7 to 2.84 milligrams to each gram of dextrose used. It would appear, therefore, that comparatively large quantities of peptone had a stimulating effect on the nitrogen-fixing properties of *Clostridium pasteurianum* and *Bacillus amylobacter*.

In 1909 Latham secured unusually high fixation with *Aspergillus niger* in nutrient solutions containing from 115.4 to 156.3 milligrams of nitrogen as ammonium nitrate for each 50 cubic centimeters of solution; however, when the nitrogen added amounted to 160.3 milligrams to each 50 cubic centimeters of solution there was a loss of nitrogen. Latham concludes that the critical point with regard to the nitrogen supply for *Aspergillus niger* is slightly below 160.3 milligrams to 50 cubic centimeters of solution. The percentage of nitrogen added to these solutions is much greater than the total percentage found in soils or in most plant tissues, and yet the nitrogen gains secured are very high for this organism.

Stahel in 1911 in studying the nitrogen-fixing power of various molds in nitrogen-free and nitrogen-containing solutions secured a decided stimulating effect by the addition of nitrogen as potassium nitrate in quantities from 0.002 to 0.016 per cent.

After a somewhat careful review of the literature bearing upon the effect of combined nitrogen on the nitrogen-fixing power of microorganisms it seems to the writer that there are not sufficient data to show that the presence of considerable quantities of combined nitrogen in the culture media reduces or destroys the nitrogen-fixing properties of organisms which show a pronounced nitrogen-fixing power in nitrogen-free solutions. On the contrary, the data obtainable seem to indicate that the nitrogen-fixing power of some organisms may be greatly stimulated by the addition of nitrogen to the culture medium.

EFFECT OF AMMONIUM SULPHATE UPON THE NITROGEN-FIXING POWER OF AZOTOBACTER CHROOCOCCUM AND AZOTOBACTER BEIJERINCKII IN NUTRIENT SOLUTIONS.

The nutrient solution employed in this experiment was prepared according to the formula recommended by Ashby, except that 15 grams of mannite to the liter were added. After a thorough mixing the solution was divided into two equal parts; one portion then received an addition of 1 gram of ammonium sulphate to the liter. The solutions were distributed in 1-liter Erlenmeyer flasks, 200 cubic centimeters being placed in each flask. After sterilization, two flasks from each lot of solution were reserved for initial nitrogen and the remaining solutions inoculated with *Azotobacter chroococcum* or *A. beijerinckii* and incubated for 18 days at 30° C. The results are shown in Table I.[1]

[1] The nitrogen determinations included in this and in the following tables were made by Dr. T. C. Trescot, of the Bureau of Chemistry, U. S. Department of Agriculture.

TABLE I.—*Effect of ammonium sulphate upon the nitrogen-fixing power of Azotobacter chroococcum and A. beijerinckii in nutrient solutions.*

Quantity of nitrogen (in milligrams) in 200 c. c. solutions.				Quantity of nitrogen (in milligrams) gained in each 200 c. c. of solution.	
At beginning of experiment.		After 18 days' incubation.			
Percentage of ammonium sulphate added.		Percentage of ammonium sulphate added.		Percentage of ammonium sulphate added.	
0	0.1	0	0.1	0	0.1
Inoculated with Azotobacter Chroococcum.					
0.56	41.54	2.25	44.07	1.69	2.61
.56	41.38	3.37	44.91	2.81	3.45
.....	.....	2.25	45.19	1.69	3.73
.....	.....	3.37	44.35	2.81	2.89
Average... .56	41.46	2.81	44.63	2.25	3.17
Inoculated with Azotobacter Beijerinckii.					
0.56	41.54	3.37	44.35	2.81	2.89
.56	41.38	Lost.	44.91	.....	3.45
.....	.....	3.09	45.47	2.53	4.01
.....	.....	2.80	Lost.	2.24	.....
Average... .56	41.46	3.09	44.91	2.53	3.45

At the conclusion of the incubation period the solutions were tested for mannite and found to contain a considerable portion of the 1.5 per cent added, but as no accurate determination of the quantity of mannite remaining in the solution was made the gain in nitrogen can not be calculated for each gram of mannite consumed. The solutions to which ammonium sulphate was added were also tested for the presence of ammonia. The method employed was to remove a few drops of the solution by means of a pipette, dilute with 10 cubic centimeters of distilled water, and treat with Nessler's reagent. Strongly positive tests were secured in every instance, thus showing that a considerable portion of the ammonium sulphate added still remained in the solution at the end of the experiment.

The quantities of nitrogen fixed for both *Azotobacter chroococcum* and *A. beijerinckii* are rather low for these organisms; however, the results seem to indicate that Azotobacter species not only fix atmospheric nitrogen in the presence of an abundant supply of combined nitrogen in the form of ammonium sulphate, but the presence of the combined nitrogen in the nutrient solution stimulates these organisms to greater activity, so that a larger quantity of atmospheric nitrogen is fixed than under conditions requiring the entire nitrogen supply to be drawn from the free nitrogen of the air.

NITROGEN FIXATION BY AZOTOBACTER ALONE AND IN ASSOCIATION WITH BACILLUS ROSSICA, WITH BOTH DEXTROSE AND CELLULOSE AS A SOURCE OF ENERGY.

Three nutrient solutions were employed in this experiment. The composition of these solutions is as follows:

Solution A.	Potassium phosphate, dibasic	gram	1
	Magnesium sulphate	do	1
	Sodium chlorid	do	1
	Calcium carbonate	do	2
	Dextrose	do	10
	Cellulose	do	1
	Tap water	c. c.	1,000

Solution B. The same as solution A with the addition of 0.05 per cent of ammonium sulphate.

Solution C. The same as solution A with the addition of 0.1 per cent of ammonium sulphate.

The results of this experiment are shown in Table II.

TABLE II.—*Nitrogen fixation by Azotobacter alone and in association with Bacillus rossica, with both dextrose and cellulose as a source of energy.*

Solution employed.	Quantity of nitrogen (in milligrams) in 500 c. c. solution after 60 days' incubation at 30° C.			Quantity of nitrogen (in milligrams) gained in 500 c. c. nutrient solution.	
	Sterile.	A. chroococcum alone.	A. chroococcum+B. rossica.	A. chroococcum alone.	A. chroococcum+B. rossica.
Solution A	30.60	34.53	39.30	4.59	9.36
	29.75	37.61	39.30	7.67	9.36
	29.47	37.33	37.05	7.39	7.11
	.....	37.61	31.75	7.67	1.81
Average	29.94	36.77	36.85	6.83	6.91
Solution B	56.70	60.63	76.91	3.08	19.36
	58.39	61.19	79.44	3.64	21.89
Average	57.55	60.91	78.18	3.36	20.63
Solution C	104.07	133.05	142.03	27.06	36.04
	119.58	133.05	143.16	27.06	37.17
	94.32	135.30	143.72	29.31	37.73
Average	105.99	133.80	142.97	27.81	36.98
	Sterile.	A. beijerinckii alone.	A. beijerinckii+B. rossica.	A. beijerinckii alone.	A. beijerinckii+B. rossica.
Solution C	104.07	133.33	140.07	27.34	34.08
	119.58	135.86	141.47	29.87	35.48
	94.32	133.89	143.16	27.90	37.17
Average	105.99	134.36	141.57	28.37	35.58

Considering, first, the results of *Azotobacter chroococcum* alone it is seen that in the nutrient solution to which no combined nitrogen was added this organism gave an average gain of 6.83 milligrams for each 500 cubic centimeters of solution. In solution B the nitrogen gained amounted to but 3.36 milligrams, which is less than half the gain shown by this organism in solution A; however, since the gain for *A. chroococcum* in solution C is almost four times as great as in solution

A it would seem that the low gains secured in solution B are due to causes other than the addition of 0.05 per cent of ammonium sulphate.

The average gain for *Azotobacter chroococcum* and *Bacillus rossica* in solution A is but little higher than for Azotobacter alone, and it is believed that the gains in either case came largely, if not entirely, from the dextrose, as *B. rossica* appeared to be unable to attack the cellulose in this solution, or if the cellulose was attacked the action was so slow that no destruction of the paper could be observed after 60 days' incubation. When 0.05 per cent of ammonium sulphate was added to the nutrient solution, a partial destruction of the paper occurred, and it is observed that the nitrogen gains in these solutions are much higher than for those in which no destruction of cellulose could be detected. In solution C, which contained 0.1 per cent of ammonium sulphate, an almost complete destruction of cellulose took place, and it is seen that the nitrogen gains are consistently higher than for either solution A or B. The results secured with *A. beijerinckii* in solution C are not materially different from those secured with *A. chroococcum* in this solution.

TABLE III.—*Nitrogen fixation by mixed cultures of Azotobacter and cellulose-destroying bacteria, with cellulose as a source of energy.*

Quantity of nitrogen (in milligrams) in 500 c. c. solution [1] after 84 days' incubation at 30° C.			Quantity of nitrogen (in milligrams) gained in 500 c. c. nutrient solution.	
Sterile.	A. chroococcum + Bacillus rossica.	A. beijerinckii + Bacillus rossica.	A. chroococcum + Bacillus rossica.	A. beijerinckii + Bacillus rossica.
92.35	101.89	102.74	8.00	8.85
94.03	104.42	103.86	10.53	9.97
94 03	101.89	103.30	8.00	9.41
95.16	101.61	102.74	7.72	8.85
.....	101.89	102.17	8.00	8.28
.....	101.89	102.17	8.00	8.28
.....	102.74	103.02	8.85	9.13
Average... 93.89	102.33	102.86	8.44	8.97

Sterile.	A. chroococcum + Bacterium fimi.	A. beijerinckii + Bacterium fimi.	A. chroococcum + Bacterium fimi.	A. beijerinckii + Bacterium fimi.
95.16	104.70	104.42	11.69	11.41
91.51	103.30	103.58	10.29	10.57
92.35	104.14	104.14	11.13	11.13
.....	104.14	104.14	11.13	11.13
.....	104.14	104.14	11.13	11.13
Average... 93.01	104.08	104.08	11.07	11.07

Sterile.	A. chroococcum + Bacterium flavigena.	A. beijerinckii + Bacterium flavigena.	A. chroococcum + Bacterium flavigena.	A. beijerinckii + Bacterium flavigena.
95.16	102.74	103.30	9.73	10.29
91.51	103.58	104.42	10.57	11.41
92.35	104.14	103.30	11.13	10.29
.....	103.29	103.30	10.28	10.29
Average... 93.01	103.44	103.58	10.43	10.57

[1] The nutrient solution employed in this experiment is the same as solution C, Table II, except that it contained no dextrose.

The table shows that the solutions containing *Azotobacter chroococcum* and *Bacillus rossica* fixed 8.44 milligrams of nitrogen on the average for each 500 cubic centimeters of solution, while the average quantity fixed by *A. beijerinckii* and *B. rossica* was but little higher. The 1 gram of filter paper added to these solutions was almost completely destroyed, so that the gains secured are for approximately 1 gram of cellulose.

The solutions inoculated with *Bacterium fimi* as the cellulose-destroying organism show gains somewhat higher than those secured from the solution containing *Bacillus rossica* as the cellulose destroyer, although much more of the cellulose remained undissolved in the solutions containing *Bacterium fimi*. It would therefore appear that the products formed by *Bacterium fimi* may be of greater value in stimulating the nitrogen-fixing properties of Azotobacter than those formed by *Bacillus rossica*.

The nitrogen gains in the solution containing *Bacterium flavigena* and *Azotobacter chroococcum* or *A. beijerinckii* are also higher than the gains secured in the *Bacillus rossica* series and but little lower than the gains in the *Bacterium fimi* series. Only a part of the cellulose added was dissolved, so that the gains can not be estimated for each gram of cellulose consumed.

CONCLUSIONS.

The addition of 0.1 per cent of ammonium sulphate to the nutrient solution stimulated the nitrogen-fixing power of *Azotobacter chroococcum* and *A. beijerinckii*.

Mixed cultures of *Bacillus rossica* and *Azotobacter chroococcum* in solutions containing both dextrose and cellulose as sources of energy gave decided gains over Azotobacter alone only when the solutions contained a considerable quantity of initial nitrogen and a destruction of the cellulose occurred.

Azotobacter chroococcum and *A. beijerinckii* in association with *Bacillus rossica*, *Bacterium fimi*, or *Bacterium flavigena* fixed quantities of nitrogen varying from 7.72 to 11.41 milligrams for each 500 cubic centimeters of solution containing cellulose only as a source of energy.

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